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(WO/2001/085927) MALARIA VACCINE AND METHODS THEREOF BASED UPON NOVEL ANTIGENIC DOMAIN OF *PLASMODIUM FALCIPARUM*

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Note: OCR Text

Malaria Vaccine and Methods Thereof Based Upon Novel Antigenic Domain of Plasmodium falciparum Field of the Invention The present invention relates to immunogenic proteins and vaccines useful for immunizing mammalian patients against infection from Plasmodium falciparum. The vaccines according to the present invention are used to introduce proteins, peptides or related material as immunogens into a patient.

Background of the Invention Malaria is one of the world's most prolific diseases, with 300-500 million new infections and 1.5-2.7 million deaths, mostly in infants or very young children, annually [World Health Organization. Control of Tropical Disease (CTD): Malaria Control. Geneva, Switzerland : WHO Office of Information, 1995; and World Health Organization. World

 malaria situation in 1992. Weekly Epidemiol Rec. 1994; 69: 309-14]. Despite intense research either to eradicate malaria or lessen its fatality and morbidity, an effective control strategy still remains to be achieved. The life cycle of the malaria parasite is multi-stage and extremely complicated [Hadley, et al., Ann Rev Microbiol. 1986; 40: 451-77]. The asexual stage of parasite development occurs in erythrocytes that undergo a re-invasion process every 48 hours. The brief window of time that the parasite is exo-erythrocytic provides an opportunity for the host to mount an immune response. The elucidation of molecular mechanism (s) responsible for the recognition and subsequent invasion of human erythrocytes by the malaria parasite is of utmost importance towards the development of a viable vaccine.

Over the years, it has become increasingly clear that such a vaccine should incorporate different antigenic regions of a variety of parasite cell surface proteins, resulting in a multi- component and multi-stage vaccine [Doolan and Hoffman, Parasitology Today, 1997 ; 13: 171- 8].

The life cycle of the malaria parasite provides several stages at which interference could lead to cessation of the infective process. In the life cycle of the malaria parasite, a human becomes infected with malaria from the bite of a female Anopheles mosquito. The mosquito inserts its probe into a host and in so doing, injects a sporozoite form of Plasmodium falciparum, present in the saliva of the mosquito.

The sporozoites which have been injected into the human host are cleared into a number of host tissue cells, including liver parenchyma cells (hepatocytes) and macrophages.

This phase is known as the exoerythrocytic cycle because at this point in the life cycle the organism has not yet entered red blood cells. After entering hepatocytes, sporozoites undergo a transformation into trophozoites, which incubate and undergo schizogony, rupture and liberate tissue merozoites. This incubation process takes approximately 7-10 days. After the incubation period, the liver or other tissue cells burst open and release numerous merozoites into the bloodstream.

Shortly thereafter, certain of these blood borne merozoites invade red blood cells, where they enter the erythrocytic phase of the life cycle. Within the red blood cells, young plasmodia have a red nucleus and a ring-shaped, blue cytoplasm. The plasmodium divides into merozoites, which may break out of the red blood cell, enter other erythrocytes and repeat the multiplication process. This period lasts approximately 48 hours.

During this same 48 hour period of the erythrocytic cycle, male and female gametocytes are formed in the red blood cells. These gametocytes also burst out of the red blood cells along with the merozoites. It is during this period that the human host experiences the symptoms associated with malaria. The merozoites which burst forth from the red blood cells live for

only a few hours in the bloodstream. The gametocytes live for several days or more in the host's bloodstream.

The gametocytes are capable of mating only in the mosquito. Thus, in order for *Plasmodiumfalciparum* to produce sporozoites for infecting a second human host, a mosquito must first bite a human host carrying gametocytes. These gametocytes mature into macrogametes, mate in the mosquito's stomach and produce a zygote. The zygote (ookinete) is active and moves through the stomach or the midgut wall. Under the lining of the gut, the ookinete becomes rounded and forms a cyst called an oocyst, in which hundreds of sporozoites develop. Sporozoites thereafter invade the entire mosquito and many of them enter the salivary glands where they are in a favorable position to infect the next host when the mosquito feeds on its blood. The life cycle thereafter simply repeats itself in another human host.

The major cell surface protein of the erythrocytic stage of *P. falciparum*, merozoite surface protein 1 (MSP-1), is considered a strong candidate for inclusion in a multi-subunit vaccine. MSP-1 is found in all species of malaria and evidence exists that people living in malaria endemic areas have MSP-1 specific antibodies in their blood stream [See, Kramer and Oberst, Am J Trop Med Hyg. 1992; 47: 429-39; Reilly, et al., Parasite Immunol 1993; 15: 513-24 ; and Gabra, et al., Bull World Health Organ. 1986 ; 64: 889-961. Furthermore, one member of this protein family, Py230, has been shown to induce a protective response in a mouse model against an otherwise lethal malarial challenge with *Plasmodium yoelii* [Holder and Freeman, Nature 1981; 294: 361-4]. The role of MSP-1 is not completely clear, but it is known to be involved in attachment, recognition and subsequent invasion of erythrocytes [Hall, et al. Mol Biochem Parasitol. 1984 ; 11: 61-80]. Therefore, a major goal is to identify a minimal region of MSP-1 that not only is highly antigenic, but that is functionally relevant and can either protect from disease or decrease parasitic load.

MSP-1 is a large polymorphic protein expressed on the parasite cell surface as a five member, multi-polypeptide complex, with each component likely playing a unique role in the complicated invasion process [Holder AA."Preventing merozoite invasion of erythrocytes." In: Malaria Vaccine Development: A Multi-immune Response Approach. Washington, DC: American Society for Microbiology Press, 1996]. There are a variety of parasite cell surface proteins involved in the parasite recognition/invasion process, but it is plausible that many of these actions may be governed by MSP-1. There are both physical events (long distance erythrocyte recognition, initial binding, re-orientation and invasion) as well as biochemical events associated with invasion [Hadley and Miller, supra]. Identification of antigenic domains within MSP-1 that are obligatory to this process may provide new targets for vaccine development.

Previous studies in our laboratory identified a region of 115 amino acids, pl 15MSP-1, as potentially binding to human erythrocytes [Su, et al., J of Immunol. 1993 ; 151: 2309-17]. pl 15MSP-1 is encoded by nucleotides 3421-3766 (West African Wellcome strain of *Plasmodium falciparum*) and the amino acid sequence (1002-116) is as follows:
 QLSFDLYNKYKLKLERLFDKKKTVGKYKMQIKKLTLLQEQLSKNSLNNPKHVLQ

 NFSVFFNKKAEIAETENTLENTKILLHYKGLVKYYNGESSPLKTLSEESIQTEDNY.

Objects of the Invention It is an object of the present invention to provide to provide immunogenic dosage forms, including vaccines which produce enhanced immunogenic activity and prophylaxis against malaria.

It is an additional object of the present invention to provide vaccines to immunize humans and other mammals against malaria.

It is another object of the present to provide a therapeutic method for producing an immunogenic response in a patient to cells of *Plasmodium falciparum*.

It is still an additional object of the present invention to provide a method for producing immunogenic responses, especially including immunogenic responses against the erythrocytic merozoite form of *Plasmodium falciparum*, using the vaccines of the present invention.

It is yet another object of the present invention to provide vaccines which provide immunogenic responses without the need for the inclusion of an adjuvant, even where the administration of the immunogen itself produces no reaction or only a weak immunogenic reaction.

Any one of these and/or other objects of the present invention may be readily gleaned from the detailed description of the

invention which is set forth herein.

Summary of the Invention The present invention relates to a malaria vaccine comprising an expression vector, preferably, a DVEE (Defective Venezuela Equine Encephalitis) viral vector system which expresses a protein corresponding to amino acids 1002-1116 (hereinafter, this 115 amino acid peptide is referred to as pl 15MSP-1) which is a specific domain of the major merozoite surface antigen (MSP-1) of the erythrocytic stage of *Plasmodium falciparum* or an immunogenic peptide fragment thereof. In certain embodiments according to the present invention, the expression vector also comprises at least one additional antigenic determinant which expresses a protein from *Plasmodium falciparum* such as a PI 9 protein or an immunogenic fragment of that protein or other proteins/protein fragments as otherwise described herein.

In the preferred virus expression vector, the DNA coding for the pl 15MSP-1 protein domain is expressed by the viral vector after administration to a patient. The pl 15MSP-1 protein or immunogenic sub-fragment which is then expressed in the patient raises a humoral and/or cell-mediated response to the erythrocytic merozoite malaria antigen, which response provides the effect of protecting the vaccinated patient from a subsequent malaria infection.

In preferred embodiments according to the present invention, the DVEE viral vector system continues to express antigen in the patient for a period of days, months or even years, thus reinforcing the immunogenic response of the patient to the expressed antigen.

The pl 15MSP-1 peptide antigen or immunogenic peptide fragment or portion thereof which is expressed by the expression vector may also comprise a signal peptide and/or an anchor peptide sequence. It is found that the addition of a signal and/or anchor peptide to the expressed pl 15MSP-1 antigen in vaccines according to the present invention unexpectedly enhances the immunogenicity to the patient of the p 115MSP-1 protein of *Plasmodium falciparum*. It is an unexpected result that the inclusion of a signal and/or anchor protein with the pl 15MSP-1 peptide can be accomplished by an expression vector system according to the present invention and the expressed peptide will produce a significantly greater immunogenic response than the p 115MSP-1 peptide alone or in combination with an adjuvant. It is also an unexpected result that the inclusion of a signal and anchor sequence in the pl 15MSP-1 peptide sequence can be expressed by the preferred viral vector system and will produce an immunogenic response which is significantly (i. e., at least about 2 times and as much as 100 times or more greater than the immunogenic response which is produced by the pl 15MSP-1 peptide which does not contain a signal or anchor peptide sequence.

Methods of inducing an immunogenic response in a patient or vaccinating a patient against a malaria infection are also contemplated by the present invention. In this method, a patient is administered an amount of pl 15MSP-1 peptide as an immunogenic fragment, thereof, alone or in combination with another merozoite surface antigen (such as PI 5, P 19 or a related peptide as described in more detail hereinbelow) or immunogenic fragment thereof in combination with a pharmaceutically acceptable carrier, excipient or additive at least once, or in certain instances, at selected intervals to provide a booster to the initial immunization or to maintain immunity in the treated patient for extended periods of time.

In a preferred method of vaccinating a patient pursuant to the present invention, a viral expression vector is capable of expressing the pl 15MSP-1 peptide of *Plasmodium falciparum* such that the patient develops an immunogenic response to the expressed peptide.

The immunogenic response generated preferably will be "substantially protective", i. e., will protect the patient from some of the more severe symptoms and physiological states of the malaria disease, including the death of the patient from malaria.

The present invention also relates to an immunogenic dosage form as a vaccine, for inducing an immunogenic response to the merozoite stage in the life cycle of *Plasmodium falciparum*. Methods of vaccinating a patient against a malaria infection are also contemplated by the present invention. In this method, a patient is vaccinated against a *Plasmodium falciparum* infection by administering an amount of a viral vector effective to establish a protective effect, preferably a long-term protective effect in a patient.. Preferably, a DVEE vector capable of expressing the pl 15MSP-1 peptide or an immunogenic peptide fragment thereof of *Plasmodium falciparum* in the patient is used in the present invention.

The present invention also relates to chimeric proteins or peptides comprising the peptide sequence corresponding to the

p1 15MSP-1 antigen (of the MSP-1 merozoite surface protein) of *Plasmodium falciparum* or an immunogenic peptide fragment thereof in combination with a signal sequence and/or anchor sequence, more preferably both a signal sequence and an anchor sequence. Other aspects of the present invention relate to chimeric proteins which comprise a peptide sequence corresponding to the p 115MSP-1 antigen or immunogenic fragment thereof and another immunogenic peptide from *Plasmodium falciparum* such as the PI 9 peptide, preferably an immunogenic peptide (or fragment thereof) which is functionally involved in non-attachment role such as processing or other functions.

Without being limited by way of theory, it is believed that chimeric peptides according to the present invention which utilize the p1 15MSP-1 peptide (which functions in *Plamodium falciparum* to enable/facilitate attachment to erythrocytes) in combination with at least one additional immunogenic peptide which functions in *P. falciparum* other than to facilitate attachment, for example, to enable or facilitate processing, growth or other functions of the parasite unrelated to attachment. Further embodiments related to chimeric protein aspects according to the present invention comprise the p1 15MSP-1 peptide or immunogenic fragment, thereof in combination with at least one additional peptide or protein (targeting peptide) which functions to target chimeric peptide to biological sites within a patient's body, for example, to maximize an immunogenic response through a cellular or humoral mediated immune response.

An alternative method of vaccinating a patient against malaria disease comprises administering an effective amount of p 115MSP-1 peptide to a patient alone or optionally in combination with at least one additional immunogenically active peptide fragment from the merozoite stage of *Plasmodium falciparum*, optionally in combination with a pharmaceutically acceptable carrier, additive or excipient. Although this method preferably avoids the administration of adjuvant along with the immunogenically active peptides (adjuvant-free), the use of adjuvant is clearly contemplated for use in certain embodiments of this aspect of the present invention.

Detailed Description of the Invention The term "patient" is used to describe an animal, including a mammal and especially including a human patient which is administered a dosage form of an expression vector or chimeric protein according to the present invention. In the present invention, the expression vector encodes for P115MSP-1 or an immunogenic peptide portion thereof and expresses the encoded protein or peptide in the patient.

The term "expression vector" is used to describe the means by which nucleic acid, including DNA, cDNA, RNA or variants thereof, more preferably DNA or RNA fragments, even more preferably RNA fragments, encoding for a specific peptide or protein, may be introduced into the patient or the patient's tissue in order to express or produce the desired protein. Such vectors include any vectors into which a nucleic acid sequence encoding for the desired P 115MSP-1 protein or immunogenic peptide fragment thereof, anchor peptide sequence and/or, signal protein sequence may be inserted (along with any required or optional operational elements) into a host organism and replicated. Expression vectors may also be used to simply produce chimeric peptide in culture for isolation. Preferred vectors are those which are capable of expressing the peptide or protein sequences in mammalian cells and whose restriction sites are well known and which contain the required operational elements for expression of the desired protein or peptide sequence. In the present invention, the vector is preferably a DVEE viral vector vector, adenovirus vector or herpes virus vector which has the capacity to infect a mammalian cell and express or synthesize proteins utilizing the host's biosynthetic mechanism. In such cases, the viral vector used for delivery should optimally be one which infects cells but which does not cause lysis due to replication (i. e., an attenuated or partially disabled virus selected from among DVEE, adenovirus, vaccinia virus and coxsackie virus, among numerous similar types).

According to the vector approach in the present invention, the vector will infect the host cells and, using the host cells biosynthetic pathways, synthesize encoded protein or peptide fragment. Any immunizing vehicle which has a detailed genetic and human use history may be used as the expression vector in the present invention and may include for example, adenoviruses, adeno-associated viruses, baculoviruses, herpes viruses, pox viruses, retroviruses and vaccinia viruses.

The preferred expression vector for use herein is a Replication Defective Venezuela Equine Encephalitis virus (DVEE).

Other expression vectors which may be used in the present invention include, for example, a vaccinia virus vector, for example, as described by Earl and Moss, Current Protocols in Molecular Biology, 1993,16.17.1-16.17.16) Smith and Moss, Gene, 1983,25, 21-28 and PCT application no. PCT/US97/01395, published as WO 97/26911, relevant portions of which are incorporated by referenced herein. However, any vaccinia or other viral vector which is used in gene therapy

and may be used in the above-described manner may be appropriate for use in the present invention, including pox viruses, as described by Paoletti, in U. S. patent no. 5,942,235, adenovirus vectors, for example, as taught in U. S. patent no. 6,033,408 and 6,057,158, bacteriophage vectors, for example, as taught in U. S. patent no. 6,054,312, recombinant alpha virus vectors, such as those taught in U. S. patent no.

6,015,686 and eukaryotic vaccine vectors, such as those disclosed in U. S. patent no.

5,942,235, all relevant portions of which references are incorporated by reference herein.

One of ordinary skill may readily modify one or more of the prior art vectors to accommodate nucleic acid fragments which express pl 15MSP-1 or immunogenic fragments thereof. In order to express the desired protein or peptide sequence, the expression vector should contain at least one promoter, at least one operator, at least one terminator codon, and any other sequences which are necessary for the efficient transcription and subsequent translation of the nucleic acid from the vector. These operational elements are well known to those of ordinary skill in the art. In preferred embodiments according to the present invention, the expression vectors will advantageously comprise at least one origin of replication which is recognized by the host organism along with at least one selectable marker and at least one promoter sequence capable of initiating transcription of the nucleic acid (preferably, DNA or RNA, most preferably RNA) sequence.

The term "vaccine" is used throughout the specification to describe a preparation intended for active immunological prophylaxis (the protective effect which is preferably long-term, i. e., at least about 6 months and preferably, at least about one year or longer). In the present invention, vaccines comprise an expression vector, preferably a DVEE virus system which expresses an antigenic protein after administration of the virus system to an animal, such as a mammal. Vaccines may also comprise chimeric peptides comprising pl 15MSP-1 or an immunogenic peptide portion thereof in combination with a signal peptide sequence and/or an anchor peptide sequence and optionally, additional antigenic peptides or immunogenic fragments thereof from Plasmodium falciparum selected from the group consisting of rap-1, resa, ebp-175 and gbp-130 from the merozoite, cspl and csp2 from the sporozoite and pfd 25 from the gametocyte, the sequences of which are attached hereto. In alternative embodiments according to the present invention, pl 15MSP-1 peptide or an immunogenic fragment thereof, is administered to a patient alone, or in combination with at least one additional immunogenic malaria peptide in combination with a pharmaceutically acceptable carrier, excipient or additive. The method of administering the vaccine (s) according to the present invention may vary and include intravenous, buccal, oral, transdermal and nasal, among others, but intramuscular or subcutaneous administration is the most common method of administration.

The term "MSP-1 protein" or "MSP-1 peptide" is used to describe the major merozoite surface protein of the erythrocytic stage of Plasmodium falciparum. This protein is found in all species of malaria. It is a large polymorphic protein expressed on the parasite cell surface as a five member, multi-polypeptide complex with each portion of the polypeptide likely playing a unique role in the invasion process. The preferred portion of MSP-1 for expression in the DVEE system system according to the present invention is a 115 amino acid unit of MSP-1 (can range from about 110 to about 120 amino acid units, referred to herein as "pl 15MSP-1" or alternatively as "P 115". This peptide is encoded by nucleotides 3421-3766 of the West African Wellcome strain of Plasmodium falciparum, plI5MSP-1 and corresponds to amino acid sequence 1002-116 as follows:

 QLSFDLYNKYKLKLERLFDKKKTVGKYKMQIKKLTLLKEQLESKLNSLNPK
 HVLQNFSVFFNKKAEIAETENTLENTKILLKHYKGLVKYYNGESSPLKTLEESIQ TEDNY The nucleotide sequence which corresponds to the above-referenced 115 amino acid peptide, as well as related nucleic acid sequences for p 15 peptides for use in the present and other amino acid and gene sequences for each of the peptides used in the present invention may be found at the website iS ://fop. wehi. edu. au/pub/biologv/who dbase, which database is available and can be used to create nucleotide constructs which are inserted into expression vectors according to the present invention. plI5MSP-1 is located in the middle of the p38 sub-unit of MSP-1, one of the 5 processed domains of MSP-1 which together form a non-covalent complex on the parasite cell surface. It is characterized by having a high isoelectric point and high lysine content. It is always found in the p38 sub-unit of MSP-1 of P. falciparum, although the amino acid sequence may vary slightly from variety to variety of Pfalciparum. The fact that pl 15MSP- 1 is a domain found entirely within one of the in vivo processed MSP-1 polypeptides, along with the results which are set forth in the present specification hereinbelow, evidences a functional role for plI5MSP-1 in the invasion of erythrocytes during the erythrocytic merozoite stage of the parasite. plI5MSP-1 is in one of only several highly conserved regions of MSP-1. In addition, this region is highly basic, with a theoretical isoelectric point (pl) of 9.44 and a large number of hydrophilic residues.

Also known as pi 15, it is generally a 115 amino acid sequence peptide (in many varieties, including FCR3, etc., the peptide is 115 amino acid units and with certain exception, the peptide may range from about 110 to about 120 amino acid units) contained within the merozoite surface protein 1 (MSP-1) of Plasmodium falciparum. The MSP-1 parasite protein is synthesized by P. falciparum as a large protein of approximately 200 thousand molecular weight. Subsequent to export to the parasite surface, the protein is processed into a number of smaller fragments that remain associated with the parasite. A major role for this complex involves recognition of and binding to the erythrocyte prior to invasion of that cell by the parasite.

As used in the present invention "p115" and "p115MSP" refer to that portion of the P38 protein of MSP-1 which is highly conserved, is highly basic (has a high concentration of lysine residues) and has a high isoelectric point. Immunogenic fragments of p115 may also be used in the present invention and these preferably comprise at least about 20 (contiguous) amino acid units, even more preferably at least about 30 (contiguous) amino acid units of the pi 15 peptide.

During the invasion process, most of the original MSP-1 is shed into the circulation of the host and a small fragment derived from the carboxyl terminus of the protein is carried into the erythrocyte. This latter fragment is termed P19, and is involved in the processing of the MSP-1 protein by the parasite protease, rather than attachment or invasion functions. In MSP-1, moving toward the amino terminus, the P19 peptide is followed by the P33 peptide and then the P38 peptide fragment. P115 is completely contained within the P38 fragment.

The entire MSP-1 protein has about 1640 or more amino acids.

P115 is typically located at amino acids 1002-116 of MSP-1, counting from the amino terminus. It is highly conserved across various strains of P. falciparum, providing further evidence for an important biological role.

Evidence for the role of P115 in erythrocyte recognition and parasite binding is as follows: -P115 produced in E. coli (see below) binds specifically to human erythrocytes.

Other proteins of similar size and/or charge distribution do not.

-The isoelectric point of P115 is very high (about 9.5). This is in contrast to that of p38 from which P115 is derived which has a pI of about 5.6. Lysine is the predominant amino acid in P 115 (27 residues). The resulting high positive charge on P 115 at physiological pH (7.4) allows for charge-based attraction to the erythrocyte surface which itself is negatively charged. In particular, the putative receptor recognition site on the erythrocyte is a domain of glycophorin A (the major red cell transmembrane glycoprotein) which has a local charge of -12.

-P115 protein is able, in a concentration-dependent manner, to inhibit the invasion of merozoites into erythrocytes.

-The amino acid sequence of PI 15 has significant similarity to the combining site region of a monoclonal antibody directed against glycophorin A. This antibody can completely block merozoite invasion of the erythrocyte. The implication of this finding is that the antibody combining site that recognizes the relevant domain of glycophorin A with high specificity resembles a site on the merozoite protein which recognizes the same structure.

-P115 itself is recognized by an anti-idiotype antibody prepared from the above-described monoclonal antibody. This latter antibody contains a mirror image of the original combining site of the monoclonal and hence is akin to the determinant on the erythrocyte surface (i. e., the region of glycophorin involved).

-Antibodies prepared in rabbit against expressed and highly purified P115 are able to completely block merozoite invasion of erythrocytes.

-The amino acid sequence of P115 is highly conserved in isolates from different geographical regions. This strongly implies a critical function for this domain since other regions of MSP-1 are variable.

Expression of P 115 has been achieved in *Escherichia coli*. Standard methods of recombinant DNA technology were employed. Purification can be achieved in several ways using well established techniques. Attachment of a polyhistidine tag to the amino terminus of the P 115 using well known methods in the art allows for direct purification on a nickel column. Alternatively, P115 can be purified directly by ion exchange chromatography taking advantage of its unusually high isoelectric point. It should be noted that for optimal expression (material to be used for booster inoculations or other commercial purpose), the DNA sequence of the P 115 in the parasite is modified by site directed nucleotide changes in the third position of selected codons. This allows for maintenance of the expressed amino acid sequence of the peptide while changing the nucleotide composition to one more closely akin to that found in *E. coli*. The same approach is used for expression in yeast or insect cells.

In general, malaria proteins are poor immunogens. Effective responses in animals generally require the use of adjuvants, most of which are unsuited for use in humans, especially in children. Therefore, alternative means of immunization may be used. Several approaches can be utilized.

For example, one approach to vaccinating a patient against malaria is to inoculate the patient directly, using either P 115 peptide or an immunogenic fragment, thereof, alone or in combination with another peptide such as P19. A similar approach is to inoculate using the DNA sequence alone. This "naked DNA" approach avoids the administration of protein directly, but its effectiveness depends on the ability of the host cell to utilize the injected DNA as a template for RNA and then protein synthesis. This method or approach may be enhanced by the inclusion of a sequence within the DNA encoding a signal and/or anchor sequence as otherwise described in detail herein or another immunogenic protein such as P19.

Another approach is to incorporate the parasite DNA sequence (which sequence may include a sequence for a signal and/or anchor sequence as otherwise described herein) into a viral or bacterial vector. The following organisms, among numerous others, may be employed for this purpose-Coxsackie virus, vaccinia virus, *Salmonella typhi* or *Salmonella typhimurium* (for oral administration). Alternative approaches which are well-known in the art and are exemplified by the teachings of, for example, one or more of United States patent number 6,130,082 (issued October 10,2000), United States patent number 5,981,182 (issued November 9,1999), United States patent number 5,720,959 (issued February 24,1998), United States patent number 5,597,708 (issued January 28,1997), United States patent number 5,504,005 (issued April 2, 1996), United States patent number 5,112,749 (issued May 12,1992), United States patent number 4,826,957 (issued May 2,1989), International Application Number WO 2000/73488 (Published December 7,2000), International Application Number WO 2000/63245 (Published October 20,2000), International Application Number WO 2000/52136 (Published September 8, 2000) and International Application Number WO 2000/46344 (Published August 10,2000), relevant portions of all of such references being incorporated by reference herein. In each case, the carrier organism must be "acquired" by a host cell and the relevant DNA sequences used for production of the PI 15 protein. This, in turn, is recognized as abnormal by the host and an immune response ensues.

Alternatively, the parasite nucleic acid sequence may be incorporated into an RNA virus or used to prepare viral replicons. The latter, using Venezuelan equine encephalitis virus, allows for the delivery of the coding sequence (as messenger RNA) to the host cell without risk of a replicative, infectious process. Signal and/or anchor sequences, as otherwise described herein, may be utilized favorably in this aspect of the present invention. In addition, the P115 may be used in a fusion protein having a second protein or peptide with targeting properties. This approach ideally includes recognition and uptake by cells of the host immune system. Preparation of viral replicons which have the capacity to invade human cells and thereupon express the parasite protein either alone or in combination with a signal and/or anchor sequence result in unexpectedly favorable immunogenic responses in patients.

Other approaches are also recognized for use in the present invention. For example, the P115 protein or immunogenic fragments thereof may be incorporated or prepared as part of another protein of known immunogenicity. This may be done, for example, with the hepatitis B surface protein which is engineered to contain PI 15 sequences while retaining the ability to form viral capsids that are highly immunogenic.

In the present invention, the preferred expressed protein is pl 15MSP-1 or any immunogenic fragment thereof, and most preferably is pl 15 MSP-1. Other immunogenic peptides or peptide fragments may also be used in combination with 115 MSP-1 or immunogenic fragment, thereof, including pl9 or an immunogenic fragment thereof, or one or more of the peptides which are disclosed by Druilhe, et al., in U. S. patent no. 6,017,538 and Inselburg, U. S. patent no. 6,024,966, or

the MSA-1 peptide or immunogenic fragments thereof, such as those disclosed in patent no. WO 97/26911, relevant portions of all such patents being incorporated by reference herein. Other preferred immunogenic peptides for use in combination with pl 15MSP-1 include for example, rap-1, resa, ebp-175 and gbp-130 from the merozoite form of *Plasmodiumfalciparum*, csp1 and csp2 from the sporozoite form of *Plasmodiumfalciparum* and pfd 25 from the gametocyte form of *Plasmodium falciparum* as well as immunogenic fragments thereof and related proteins and immunogenic fragments thereof, the sequences of which are attached hereto or may be readily obtained from the SWISSPROT database at <http://www.expasy.ch/csJi-bin/sprot-search-de> using *plasmodium falciparum* as search terms, relevant portions of which including the peptide fragments are incorporated by reference herein.

Quite surprisingly, the P115 antigen is substantially more antigenic than are other non-P1 15 peptide fragments of the MSA-1 peptide. For example, the P19 fragment (discussed above) has been extensively studied as a candidate antigen (NIH, Mill Hill, etc.).

In our experiments, antibodies were prepared against recombinant P19 (prepared for possible use in human trials) and these were compared directly to the P115 antibodies. Quite surprisingly, the antibodies generated against P115 were four times as effective (requiring 4 fold less antibody to achieve the same level of inhibition of merozoite invasion) as were the antibodies generated against P19. This is an unexpected result.

In addition to the heightened immunogenicity of P 115 compared to P 19, a combination of P115 and P1 9 peptides provide a synergistic result against the parasite.

While not being limited by way of theory, it is believed that the synergistic immunogenicity (enhanced immunogenicity and more effective vaccination) which results from the inclusion of P 115 and P1 9 peptides or immunogenic fragments thereof in certain aspects of the present invention occurs because of the activity of the P1 9 peptide antibodies in preventing the normal processing of the MSP-1 by the parasite protease, while the P115 peptide antibodies directly inhibit invasion of the erythrocytes. Combinations of both of these antigens make especially effective anti-merozoite vaccines.

Although a merozoite vaccine according to the present invention will not prevent infection, it will reduce the likelihood that an individual will contract malaria and its use in hyper-endemic areas is especially effective since continual natural boosting of the immunogenic response will occur. In addition, depletion and/or elimination of merozoites in the human host will result in a lessening or even elimination of gametocyte production, thus reducing transmission and ultimately, infectivity of the vector population.

The term "immunogenic fragment" refers to a peptide fragment from a larger peptide fragment which exhibits immunogenicity (produces antibodies) in the *in vitro* invasion assay as described in the specification (see examples section) and as otherwise described in Hui and Siddiqui [Exp. Parasitol., 1987; 64: 519-22]. A percent inhibition of invasion of at least about 25% (more preferably, at least about 50%, even more preferably at least about 75%) in that assay is considered immunogenic for purposes of this invention.

Peptide fragments which exhibit levels of inhibition of at least about 90% or more are particularly preferred for use in the present invention.

The term "chimeric protein", "chimeric peptide" or "chimeric peptide sequence" is used to describe the non-natural peptide sequences according to the present invention which comprise the pl 15MSP-1 protein or peptide and at least one additional peptide sequence and can include, for example, an anchor peptide and/or a signal peptide incorporated within the same molecule. As noted by the use of this term in the present invention, chimeric peptides generally are synthetic peptides produced by an expression vector which contain a desired target protein or peptide (either P1 15MSP-1 or an immunogenic peptide portion thereof) in combination with another peptide sequence (either an anchor or signal peptide sequence).

Any expressed peptide which substantially corresponds to the pl 15MSP-1 peptide or an immunogenic peptide fragment, thereof may be used in the present vaccines. Of course, expressed peptides corresponding to the pl 15MSP-1 peptide or an immunogenic peptide fragment thereof in combination with a signal sequence or anchor sequence may also be used preferably in the present invention.

The term "signal peptide" "signal sequence" or "signal protein" is used to describe a 7-30 unit amino acid peptide sequence, preferably about a 15-26 unit amino acid peptide sequence, which is generally found at or near the N-terminus of the expressed protein or peptide which is used in the present invention in order to substantially enhance the biological activity of the protein or peptide expressed in the patient according to the present invention.

Signal sequences generally contain hydrophobic peptide sequences of between about 7 and 30 amino acid units, more preferably, about 15 to 26 amino acid units, even more preferably about 16 to 24 amino acid units and most preferably about 18 to 20 amino acids units appear to be essential for the targeting of protein chains (generally, secretory proteins) to membranes within the cell. These hydrophobic sequences are of sufficient length to cross the lipid bilayer of the cell membranes. Signal sequences serve as organizers for the cellular traffic of macromolecules. These proteins are believed to play a central role in the translocation of polypeptide chains across membranes. In the present invention, the incorporation of a signal protein sequence at the amino terminus of the protein or peptide sequence expressed by the vaccinated patient is associated with the substantial enhancement in the biological activity (including the therapeutic effect of immunogenicity) associated with the expressed protein or peptide. In the present invention, signal sequences which are known in the art may be used in the present invention. For example, although it may be possible to utilize yeast or lower trophic order signal sequences, clearly mammalian signal sequences are preferred for use in mammals and the specific species signal sequences are most preferred for use in the desired mammalian species to be treated. Thus, in providing for an expressed protein or polypeptide in humans, a human signal sequence is most preferred.

Signal sequences for use in the present invention generally contain three regions, a first or c region at the carboxy end of the peptide (which serves as the cleavage site for a signal peptidase enzyme), comprising about 5 to 7 amino acid residues which tend to be highly polar but uncharged; a second or h region which is N-terminal to the c region, generally about 7 to 13 amino acid residues in length and highly hydrophobic (comprised primarily of Leu, Ala, Met, Val, Ile, Phe, and Trp amino acids, but may contain an occasional Pro, Gly, Ser or Thr amino acid residue); and a third region or n-region of highly variable length and composition, but generally carrying a net positive charge contributed by the N-terminus (negative charges contributed from acidic residues are also known) and any charged residues. Between the c region and the h region are between 1 and 3 amino acid residues which tend to be small and uncharged (Ala, Gly, Ser, others). Synthetic homopolymeric h regions comprised of amino acids selected from the group consisting of leucine, isoleucine, phenylalanine, valine, alanine and tryptophan, preferably leucine, isoleucine and phenylalanine may be used in the signal proteins according to the present invention. See generally, von Heijne, European Journal of Biochemistry, (1983), 133, pp. 17-21.

The signal sequences which are used in the present invention preferably encompass eukaryotic signal sequences, even more preferably mammalian sequences and even more preferably human sequences, preferably between 7 and 30 amino acid units in length, preferably between 15 and 26 units, more preferably between about 16 and 26 amino acids, even more preferably between 18 and 20 amino acid units. In the present invention, the c region of the signal peptide should be more polar and the boundary between the h and c regions between residues-5 and -6, or -7 or -8 (counting from the position of cleavage of the signal sequence-i. e., the first amino acid of the mature or expressed protein or peptide is +1) is between 1 and 3 amino acid residues which tend to be small and uncharged (Ala, Gly, Ser, others). Position preferences in the h/c for amino acids are as follows: -10 most preferably leucine or alternatively, isoleucine, valine, alanine, or phenylalanine; -9 most preferably leucine, alternatively, isoleucine, alanine, valine, phenylalanine; -8 most preferably leucine, alternatively isoleucine, alanine, valine, glycine, phenylalanine; -7 most preferably alanine, alternatively, leucine, isoleucine, valine, phenylalanine; -6 most preferably valine, alternatively leucine, valine, isoleucine, phenylalanine, alanine; -5 most preferably proline, alternatively glycine, alanine, leucine, valine; -4 most preferably glycine, alternatively proline, leucine, alanine, valine; -3 most preferably alanine, alternatively valine; -2 most preferably leucine, alternatively phenylalanine; -1 most preferably alanine, alternatively glycine.

In the signal sequences used in the present invention, the h region may vary in length as well. The n region is polar, contains positively charged amino acids (predominantly lysine and arginine) and varies with the overall length of the signal peptide as described above. The c region extends from residues-1 to -5 of the signal peptide/expressed or mature protein. In terms of location of the c, h and n regions, the c region is N-terminus to the expressed or mature protein, the h region is N-terminus to c region (with a 1-3 amino acid boundary between the c and h region) and the n region is a positively charged N-terminus to the h region. In sum, the n region is variable in length and generally positively charged (with a preferred charge of +2), the h region is hydrophobic and variable in length and the c region preferably contains about five (5-7) generally polar amino acids.

The end of the hydrophobic domain (i. e., the boundary between the hydrophobic residues enumerated above) should preferably be at positions 6/-5. Overall, the signal sequence should comprise a 5 to 10 unit residue initial sequence (beginning with methionine) followed by at least a seven residue sequence (as described above) and an additional amino acids from 1 to 10 residues in length. A typical sequence for the region noted about is: ILLLLAV.

The signal sequence used should be characteristic of the cell type used for expression of the protein. Thus, in veterinary applications, the signal sequence most preferably used should be that of the animal to be treated. Often a signal sequence which is mammalian in character is acceptable for human applications. Most mammalian signal sequences will have significant efficacy in expressing proteins or peptides in other mammalian cells, including human cells. Human signal sequences are preferably used in the present invention.

In the present invention, the following signal peptide DNA sequences may be used: ATG AAGATCATAT TCTTTTATG TTCATTCTT TTTTTATTA TAAATACACAATGTG; and ATG AAGATCATAT TCTTTTATG TTCATTCTT TTTTTATTA TAAATACACAATGTGTAACA CATGAAAGTT ATCAAGAACT TGTCAAAAAA CTAGAAGCTTAGAAGATGC AGTATTGACA GGTTATAGTT TATTCAAAAA GGAAAAAAATGGTATTAAATG AA.

The amino acid sequences corresponding to the above-described signal peptide DNA sequences are:
MKIIFLCSFLFFIINTQC ; and MKIIFLCSFLFFIINTQCVTHESYQELVKKLEALEDALGYSLFQ KEKMLVNE.

The term "anchor protein" or "anchor peptide sequence" is used to describe proteins or peptides which are anchored to the external surface of the plasma membrane generally by covalent bonding to glycans containing phosphatidyl inositol. These structures to which the anchor protein or peptide is bonded are often referred to as glycosyl phosphatidyl inositol or GPIs. In all cells, anchor proteins covalently bonded to GPIs are found on the external face of the plasma membrane of cells or on the luminal surface of secretory vesicles.

In the present invention an "anchor protein" or "anchor peptide" comprises a peptide sequence preferably of about 15-35 residues in length which is generally expressed at the carboxy-terminus of the protein or peptide expressed by the expression vector according to the present invention (3'end of the DNA sequence expressing the desired protein or peptide and carboxyl terminus of the expressed protein or peptide).

In the present invention, many of the proteins or peptides which are expressed in the patient and in particular, the immunogenic proteins or peptides of vaccines according to the present invention which are expressed in the patients produce a biological or immunogenic response in the patient which is substantially enhanced when an anchor peptide is incorporated at the carboxy terminus of that protein or peptide. The inclusion of a signal protein at or in the proximity of the N-terminus, in addition to the anchor peptide at the carboxy-terminus of the expressed protein, is associated with an unexpected enhancement in the biological effects of the expressed protein. This is especially true where the expressed protein is antigenic or immunogenic in nature.

In preferred embodiments according to the present invention, the carboxy-terminus of the expressed protein or peptide residue (generally, at least the p1 15MSP-1 peptide or immunogenic fragment thereof) is modified by attachment of a glycolipid anchor, which serves to anchor the modified protein or peptide to the cell surface. The peptide residue to which the GPI anchor is added is always one of small amino acids, such as glycine, aspartic acid, asparagine, alanine, serine and cysteine. These occur at the carboxyl terminus of the protein/peptide of interest and thus can be specified by inclusion of the appropriate codons in the DNA fragment to be added to the cDNA sequence specifying the protein/peptide of interest. In addition, the two residues downstream of the anchor addition site are usually small.

The cleavage/anchor addition site resides in a domain of three small amino acid residues, although the central of the three residues has less stringent steric requirements. In order to be certain that functionally or immunologically important amino acids at or near the carboxyl terminus of the protein/peptide target are not compromised, several additional amino acids (preferably, polar ones such as lysine or arginine as well as threonine, alanine and proline) to make up a total of up to 10 residues are inserted in such an orientation so that the small, polar segment is at the carboxyl terminus. The remainder of the addition signal sequence will contain from 15 to 35 amino acids with a hydrophobic domain at the extreme carboxyl terminus. This domain should extend for 15-25 amino acids and will include amino acids such as valine, leucine, isoleucine, alanine, phenylalanine, but may also contain proline and glycine as well as tryptophan. A typical such

sequence is as follows: TACDLAPPAGTTD AAHPGRSVVPALLPLLAGTLLLETATAP The small sequence is in bold face with the left portion representing the terminus of the protein and the D residue the site of GPI addition. The right hand portion is that cleaved during GPI addition with the underlined sequence indicating the hydrophobic terminus.

In the present invention, the anchor peptide may have a cleavable N-terminal sequence, which directs the peptide to the endoplasmic reticulum and the cellular trafficking pathway where the GPI anchor is added. As described above, the anchor peptide also has a predominantly hydrophobic sequence at the extreme carboxy terminus which generally ranges in size from about 15 to about 35, more preferably about 15 to 30, and even more preferably about 15 to 25 amino acid residues, signals the addition of the GPI anchor and is cleaved off concurrent with GPI addition. It is the hydrophobicity rather than the sequence itself which is important for anchor addition. Essentially any hydrophobic amino acid sequence of at least about 15 to about 35, more preferably about 15 to 30 amino acid residues would be capable of directing the addition of a GPI anchor-. Anchor addition is generally a transamidation reaction in which the free ethanolamine amino group of the GPI precursor attacks (by way of nucleophilic addition) a peptide bond at the target amino acid, which becomes the C-terminal amino acid.

Generally, in the expressed anchor peptide sequence, just upstream of the hydrophobic sequence to which the GPI anchor is added is a hydrophilic spacer (usually about 5-10 residues) which contains hydrophilic amino acids. The residue to which the GPI anchor is added (the "anchor addition site") is an amino acid residue within this hydrophilic spacer selected from the group consisting of glycine, aspartic acid, arginine, asparagine, alanine, serine and cysteine. In addition, the two residues downstream from the anchor addition site are also usually small amino acid residues apparently to minimize steric hindrance at the anchor addition site.

Preferably, the GPI portion is preassembled and added as a single unit to a specific amino acid residue near the carboxyl terminus of the expressed protein or peptide. Thus, the carboxyl terminal region may be characterized by the presence of a C-terminal signal peptide which is preferably ten to thirty amino acids in length and provides the information needed to add the GPI anchor. The actual amino acid residue to which the GPI structure is attached is called the omega site and this residue should be glycine, alanine, cysteine, serine, asparagine or aspartic acid. The omega +1 site (towards the carboxyl terminus of the expressed, unprocessed protein) preferably is selected from glycine, alanine, cysteine, serine, asparagine, aspartic acid, glutamate and threonine. The omega +2 site is alanine or glycine. The omega +2 site is followed by a hinge or spacer of ideally 5 to 7 amino acids that preferably contains charged amino acids and proline; this is followed in turn by a preferably hydrophobic sequence of amino acids which terminate the carboxyl signal peptide.

The overall structure of the anchor peptide may be summarized as a 15-35 amino acid peptide at the carboxyl terminus of the expressed protein or peptide. This anchor peptide sequence (reading from the terminus towards the amino end) begins with a hydrophobic stretch of amino acids of variable length, followed by a sequence of preferably 5-7 amino acids which contains charged residues, followed by three amino acids (either glycine or alanine at the omega +2 site); any of glycine, alanine, cysteine, serine, asparagine, aspartic acid, glutamate and threonine at the +1 omega site; and any of glycine, alanine, serine, cysteine, aspartic acid or asparagine at the omega site.

It is noted that in the present invention, while the signal peptide sequence is generally found at the N-terminus of the expressed peptide (directly at the N-terminus or removed as much as 1,000 or more amino acids from the N-terminus) and the anchor peptide sequence is generally found at the carboxy-terminus of the expressed protein or peptide, the signal peptide may be found at or near the carboxy terminus of the expressed target protein or peptide.

In the present invention, anchor sequences which are known in the art may be used in the present invention. For example, although it may be possible to utilize yeast or lower trophic order anchor sequences, clearly mammalian anchor sequences are preferred for use in mammals and the specific species signal sequences are most preferred for use in the desired mammalian species to be treated. Thus, in providing for an expressed protein or polypeptide in humans, a human anchor sequence is most preferred.

In the present invention, the following anchor peptide DNA sequence is preferably used: TTCTTAGGAA TATCATTCTT ATTAATACTC ATGTTAATAT TATCCAGTTT CATTAA.

The amino acid sequence corresponding to the above-described anchor peptide DNA sequence is :

FLGISFLLILMLILYSFI.

It is noted that in the present invention, while the signal peptide sequence is generally found at the N-terminus (directly at the N-terminus or somewhat removed-by as much as 1,000 or more amino acids from the N-terminus) and the anchor peptide sequence is generally found at or in the proximity of the carboxy-terminus of the expressed protein or peptide, the signal peptide may be found at or near the carboxy terminus and the anchor peptide may be found at or near the N-terminus of the expressed target protein or peptide.

The term "effective amount" refers to an amount or concentration of expression vector (generally, a viral vector and most preferably, a DVEE viral vector which is administered to the patient which is effective to produce a protective immune or therapeutic response with respect to the disease malaria. In general, an effective amount of the viral vector which is administered to a human patient will vary depending upon a number of factors associated with that patient, including whether the patient previously has been exposed to *Plasmodium falciparum* before. An effective amount of the viral vector can be determined by varying the dosage of the product and measuring the resulting cellular and humoral immune and/or therapeutic responses, prior to administration. In general terms, in humans, this amount represents approximately 10⁴ to about 10⁷ plaque forming units, preferably about 1 X 10⁶ to about 5 X 10⁶ plaque-forming units (determined by assay, as described in PCT Applicant No.

PCT/US97/01395, published as WO/26911). It is noted that the above described range of administered viral vector is chosen to enhance the likelihood of eliciting an immunogenic response (vaccinating the patient for a long period of time) without causing a malaria infection in the vaccine recipient.

In the case where the p1 15MSP-1 peptide or an immunogenic fragment thereof, or a chimeric peptide containing p1 15MSP-1 peptide or an immunogenic fragment thereof is administered instead of the expression vector in order to facilitate an immunogenic response, the amount of peptide (chimeric or non-chimeric) administered will be an amount or concentration of the peptide to produce a protective immune or therapeutic response with respect to the disease malaria. While this amount may vary over a considerably wide range, depending upon the immunogenicity of the peptide chosen, generally the amount of peptide administered ranges from about 0.01 micrograms (10 nanograms) to about 250 micrograms, more preferably about 0.1 microgram to about 100 micrograms, even more preferably about 1 microgram to about 25 micrograms within this range.

The present invention contemplates, as an alternative and in some cases, a preferred embodiment, the incorporation of both a signal peptide and anchor peptide along with the p1 15MSP-1 peptide into the expressed peptide. This is found to be advantageous in producing an immunogenic response to the P115MSP-1 peptide. The signal peptide sequence is generally incorporated into the immunogenic peptide at or near the amino end of the p1 15MSP-1 peptide (or a related antigenic peptide which may also be expressed in combination with p1 15MSP-1 by the expression vector) and the anchor peptide is generally incorporated at or near the carboxyl end of the p1 15MSP-1 peptide. The immunogenic peptide is expressed by the viral vector accordingly and will contain a signal peptide sequence and/or an anchor peptide sequence. Thus, in the present invention, the signal and anchor peptides are preferably expressed at the amino and carboxy terminus of the expressed p1 15MSP-1 peptide, respectively. Generally, the signal peptide sequence is located upstream from the p1 15MSP-1 peptide and the anchor peptide is downstream from the p1 15MSP-1 peptide.

In the method of preparing the viral vector which leads to expression of p1 15MSP-1 peptide or an immunogenic fragment thereof containing a signal and/or anchor peptide, by the vaccine recipient, any method which is capable of incorporating a sequence of DNA containing genetic material for the expression of the p1 15MSP-1 peptide and optionally, a signal peptide and/or an anchor peptide, may be used. The method which may be used in the present invention is well known in the art. Accordingly, in the present invention, a DNA sequence containing the genetic code for the p1 15MSP-1 peptide to be expressed is obtained by chemical synthesis or other means such as biochemical isolation of available P115MSP-1 DNA sequences and incorporated into a cloning plasmid (for example the following cloning vectors: pBR322, pGEM3z, pSP70, pSE420, pRSET, lambdaZAP, all commercially available, among numerous others). The appropriate DNA sequence is cloned, isolated, for example, using agarose gel electrophoresis and then incorporated into an amplification vector and amplified by a standard polymerase chain reaction technique for a sufficient number of cycles to obtain a desired quantity of DNA (depending upon the amount of DNA desired, from about 5 cycles to about 40 cycles or more). A signal peptide sequence and/or anchor peptide sequence may be incorporated into a vector containing the p1 15MSP-1 peptide and, after identification (selection and screening) of the appropriate DNA fragments in positive clones by PCR and

endonuclease digestion, amplified accordingly using the same techniques.

After amplification, the DNA is incorporated into a transfer vector and transfected with eukaryotic cells, for example, monkey kidney cells (BSC-1 cells), and with wild-type virus to produce recombinant DVEE viral vector. The recombinant DVEE viral vector replicon is then purified before amplification. After amplification and in some cases further purification, the recombinant DVEE viral vector in vaccine dosage form is then administered directly (i. e. without being incorporated into somatic cells further preparation or mito an animal as an immunogenic dosage form which expresses p1 15MSP-1 peptide or an immunogenic portion thereof, preferably, in combination with a signal peptide and/or an anchor peptide. In alternative embodiments, rather than administer the expression viral vector in vaccine dosage form to a patient, somatic cells or other cells from the patient may be removed and transfected with the viral vector and the transfected cells may be used to treat a patient patient. Thus, the methods which are set forth in U. S. patent nos. 6,054,288 and 6,9048,729, relevant portions of which are incorporated herein may be used as alternatively approaches.

Alternatively, once a nucleic acid sequence encoding immunogenic chimeric protein is present in a suitable expression vector, the expression vector may be used for the purpose of expressing the immunogenic chimeric protein in a suitable eukaryotic cell system, for example, to promote the production of the desired peptide sequence outside of the host animal. Such eukaryotic cell systems include, for example, HeLa, L929, T2 or RMA-2, preferably T2 or RMA-S. In this method, the cells which contain the expression vector (s) are grown and then lysed in order to isolate synthetic peptides which contain the desired protein or peptide sequence in combination with the anchor peptide sequence and/or the signal sequence. The isolated peptide sequence may then be used directly as a therapeutic or immunogenic dosage form. Alternatively and preferably, the expression vector may be administered directly to the patient where it will express the desired protein or peptide and anchor sequence and render the intended therapeutic or immunogenic effect on the patient.

The expressed protein may be obtained from cell culture after the cells are lysed by standard protein purification procedures known in the art which may include, among others, gel electrophoresis, affinity and immunoaffinity chromatography, differential precipitation, molecular sieve chromatography, isoelectric focusing and ion-exchange chromatography. In the case of immunoaffinity chromatography, the protein or peptide may be purified by passage through a column containing a resin to which is bound antibodies which are specific for at least a portion of the protein or peptide.

The expressed protein or peptide containing a signal peptide sequence and/or an anchor peptide sequence, which is obtained from cell culture, may be administered in pure or substantially pure form to a patient in need of such therapy by purifying the crude lysate from cell culture. Preferably, the expressed protein is administered in pharmaceutical dosage form as a composition or formulation comprising an immunogenically effective amount of the expressed protein containing anchor peptide sequence and/or signal peptide sequence, in combination with a pharmaceutically acceptable additive, carrier or excipient. The formulations may be delivered in unit dosage form prepared by known methods in the art.

The amount of expressed protein or peptide administered will vary depending upon the pharmokinetic parameters, severity of the disease treated or immunogenic response desired.

Of course, dosages will be set by the prescribing physician considering relevant factors including the age, weight and condition of the patient including, in the case of immunogenic dosage forms, whether the patient has been previously exposed to the microorganism responsible for the disease to be vaccinated against as well as the release characteristics of the expressed protein from pharmaceutical dosage forms of the present invention.

The amount of the expressed protein which is administered according to the present invention comprises an amount effective to produce the intended effect, i. e., to obtain an immunogenic response in the patient which provides a substantially protective effect against malaria.

Alternatively and preferably, the vaccine which is administered according to the present invention comprises an amount of an expression vector, preferably, a recombinant DVEE viral vector effective to express sufficient P115MSP-1 peptide to provide an immunogenic response in a patient. Preferably, the P115MSP-1 peptide or an immunogenic peptide sequence thereof is combined with a signal and/or anchor peptide to substantially increase the immunogenicity of the expressed P115MSP-1 peptide compared to P115MSP-1 peptide which does not contain a signal and/or anchor peptide. The immunogenic response provides a protective effect against the merozoite stage of malaria.

The present vaccine can be injected as is, or for convenience of administration, can be added to a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable carriers will be apparent to those skilled in the art, and include water and other polar substances, including lower molecular weight alkanols, polyalkanols such as ethylene glycol, polyethylene glycol, and propylene glycol as well as non-polar carriers.

Dosages of recombinant DVEE viral vector or chimeric protein or peptide according to the present invention which are coadministered with carriers will often be about the same as the amount administered alone (in the absence of coadministration). Of course, dosages will be set by the prescribing physician considering relevant factors including the age, weight and condition of the patient including whether the patient has been previously exposed to *Plasmodium falciparum* and the release characteristics of the DVEE viral vector from pharmaceutical dosage forms of the present invention.

In the malaria vaccine aspect of the present invention, the dose of DVEE viral vector will depend upon the form in which it is administered. For example, the vaccine will generally contain a concentration of virus ranging from about 10⁴ to about 10⁷ plaque forming units, preferably about 1 X 10⁶ to about 5 X 10⁶ plaque-forming units, depending upon the desired levels of expressed immunogenic protein. Thus, the concentration or amount of viral vector included within the present vaccine will generally fall within this range; however, the amount of viral vector used in any vaccine form will depend upon the strength of the immunogenic response elicited.

In determining the amount of viral vector in a given vaccine dose, the following method may be used. In certain vaccine dosage forms, standard pharmaceutical carriers as described above may be included. The ratio of virus included in the vaccine will depend on the chemical nature, solubility, and stability of the virus, as well as the dosage contemplated.

For parenteral administration or injection via such parenteral routes as intraperitoneal, intramuscular, subcutaneous, intramammary or other route, sterile solutions of the DVEE viral vector are prepared. Vaccines according to the present invention may also be administered intravenously. Preferably, the vaccines according to the present invention are administered via a subcutaneous route.

The dosage of the vaccine employed and the treatment schedule would follow practices normally employed for other vaccination or therapeutic regimens wherein this general method of treatment is employed. It is not anticipated that more than one dose of vaccine initially would be required, but the possibility of providing booster doses is anticipated. Preferably, the dosage schedule for immunization against malaria involves the subcutaneous injection of at least about 1 X 10⁶ plaque-forming units of DVEE viral vector.

In the immunogenic method according to the present invention, a human patient is administered with an effective amount of DVEE viral vector such that expressing the pI 15MSP-1 peptide or an immunogenic peptide thereof, preferably in combination with a signal and/or anchor peptide. Alternatively, an immunologically effective chimeric peptide comprising the pI 15MSP-1 peptide or an immunogenic portion thereof in combination with a signal peptide and/or anchor peptide will be administered. In certain instances, an additional boost of DVEE viral vector or peptide may be given to promote the immunogenic response.

Additional doses of vaccine may be provided to boost the initial inoculation, if needed.

The present inventors undertook a study to determine whether pI 15MSP-1 exhibited any role in parasite invasion. The goal of the study which lead to the present invention was to elucidate any potential function that pI 15MSP-1 may play in the parasite invasion process and to define whether or not this region could be used in a vaccine as an antigenic target to elicit a host immune response. Using a bacterial over-expression system, we synthesized and purified histidine tagged, recombinant pI 15MSP-1. Polyclonal antibodies to this region were generated and their ability to inhibit parasite invasion was investigated. We showed in our results that not only is this region highly antigenic, but that antibodies to this region are surprisingly effective in preventing parasite invasion, *in vitro*.

As part of the study, we further investigated the role of the pI 15MSP-1 itself, showing that this protein is capable of specific binding to human erythrocytes and that this interaction can abrogate parasite invasion, *in vitro*. Furthermore, the pI 15MSP-1/erythrocyte interaction occurs with EnA (-) erythrocytes (i. e., erythrocytes devoid of glycophorin A, a major erythrocyte

receptor for the invading parasite [Pasvol and Jungery, "Glycophorin and red cell invasion by *P. falciparum*." *Malaria and the Red Cell*. Pitman, London (Ciba Foundation Symposium 94) 1983 ; 174-95]), suggesting a potentially novel MSP-1-erythrocyte interaction. Finally, we show that pI15MSP-1 is recognized by sera of individuals from Cameroon, a malaria-endemic region of Africa, indicating that antibodies to this domain, or a portion thereof, are made in naturally infected individuals. The findings described in this study provide evidence for the involvement of pI15MSP-1 in merozoite invasion.

The following examples are provided for purposes of illustration only and are not to be viewed as a limitation of the scope of the invention.

EXAMPLES Materials and Methods Plasmid construction and gefze atnplification Plasmid pME-14, containing the C-Terminal region of the MSP-1 gene from the West African Wellcome (Lagos) strain of *P. falciparum*, was generously donated by Holder, et al. and used as a template for PCR [Holder, et al., *coli Parasite Immunol* 1988 ; 10: 607-17]. The gene fragment corresponding to DNA sequence 3421-3766 (the amino acid sequence 1002- 1116) was amplified (94 C melting, 72 C extending and 50 C annealing) using oligonucleotides designed with unique restriction sites, 5'-GCCTCGAGCAGTTATCATTGATTATATAAT-3' (sense oligonucleotide, engineered with an Xhol (New England Biolabs) restriction site) and 5'- GCGGTACCATATTATCTTCTGTTGAATTGAT-3' (antisense oligonucleotide, engineered with a KpnI (New England Biolabs) restriction site). The PCR product was gel purified using Sephadex BandPrep Kit (Pharmacia) and sub-cloned, in frame, into a histidine tag over-expression vector from Invitrogen (pRSET B). Bacterial colonies were screened for positive clones. DNA was isolated, confirmed by sequencing and used to transform pLys BL21 E. Coli for over-expression. Sequencing was done using an ABI 377 DNA autosequencer (Applied Biosystems) based on fluorescence methodology using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer).

Protein over-expression and purification Bacteria containing the over-expression vector were grown overnight in Luria-Bertani Medium (LB) + ampicillin (100 ug/ml). The culture was diluted 1: 20, grown to an OD600 of 0.6 and induced with 1 mM IPTG (Sigma) for 3 hours. Protein from the induced culture was purified under denaturing conditions according to the protocol in the Invitrogen purification manual. Briefly, induced bacteria were lysed with a 6M guanidium lysis buffer and a 5 minute pulse of sonication. The lysate was purified on a pre-washed Ni-NTA-Agarose nickel column (Qiagen), washed with a pH gradient (pH 6.0-4.95) and eluted with a pH bump (pH 4.0). Purified protein was collected and dialyzed overnight against 2 liters of PBS (pH 7.4) at 4 C. Purity of protein was determined by Coomassie (Coomassie Brilliant Blue R-250, Gibco-BRL) staining of an SDS polyacrylamide gel. Concentration was determined using a BCA Protein Assay Reagent Kit (Pierce).

Antibody preparation 200 ug of pI 15MSP-1, in a volume of 225 ul, was vortexed (for 1 minute) with 225 ul Titermax (Vacek, Inc.) adjuvant and injected sub-cutaneously into a New Zealand white rabbit at four sites. The rabbit was boosted (200 gag protein + Titermax adjuvant) after 3 weeks and serum was harvested 10-14 days post boost. Titer was determined by ELISA (procedure follows) and IgG antibodies were purified by protein A-agarose chromatography using a MAPS 2 kit (Biorad). Antibody was dialyzed overnight against 2 liters PBS (pH 7.4) and concentration was determined by optical density. Final concentration was 3.1 mg/ml in PBS (pH 7.4).

ELISA to measure titer of pI 15MSP-1 A 96 well microtiter plate (IMMULON 2, Dynatech Laboratories) was coated with 100 gl of pI 15MSP-1 (15 Igg/ml) in PBS (pH 7.4) and incubated overnight at room temperature. Wells were blocked with 200 ul 3% BSA in PBS (pH 7.4) for 5 hours at room temperature, washed 3 times with PBS (pH 7.4), incubated with varying dilutions of p 15MSP-1 antibodies for 2 hours at room temperature, washed 3 times with PBS (pH 7.4), incubated with 100 sul secondary antibody (donkey anti-rabbit Ig, horseradish peroxidase linked whole antibody, 1: 2000 (Amersham)) for 2 hours at room temperature, washed 4 times with PBS (pH 7.4), incubated with 100 1li 3, 3', 5,5'-tetramethyl benzidine (TMB) liquid substrate system (Sigma) for 30 minutes at room temperature, supplemented with 100 u. 10. 5M sulfuric acid and read on a 96 well plate reader at 450 nm. Values were normalized to background (substrate alone).

In vitro culture of *P. falciparum* *P. falciparum* was grown based on a protocol described by Siddiqui and Palmer [Siddiqui and Palmer, "Propagation of malaria parasites in vitro." In : "Advances in Cell Culture." New York: Academic Press, 1981 ; 1: 183-212]. *P. falciparum* isolates (FCR-3 strain) were cultured in human O+ erythrocytes in RPMI medium (5.94% HEPES, 0.05% hypoxanthine, 0.001% PABA, 0.24% sodium bicarbonate, 0.005% gentamicin and 10% human serum (heat inactivated prior to use)) at 37 C, in 90% Nitrogen, 5% Oxygen, 5% Carbon Dioxide. Cultures were maintained at 1-2% parasitemia and 2% hematocrit. Prior to invasion assays, cultured parasites were synchronized twice by treatment with 5%

sorbitol [Lambros and Vanderberg, Journal of Parasitology 1979; 65: 418-20]. The parasite culture utilized in Fig. 1, panel A was harvested in late trophozoite/early schizont stage at 14% parasitemia. The culture was washed extensively with PBS (pH 7.4) and resuspended in 1X SDS loading buffer [Sambrook, et al., Molecular Cloning : a laboratory manual. 2na ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press 1989].

Invasion assay and quantitation of parasites Invasion assays were based on protocols described by Hui and Siddiqui [Exp Parasitol. 1987; 64: 519-22]. Synchronized parasites (1-2% parasitemia) were seeded at a 3.0% hematocrit in an adjusted volume of 170 ul. At early ring stage, antibody was added in varying concentrations at a constant volume of 30 III. All samples were in PBS (pH 7.4) and compared to controls of 30 ul of PBS (pH 7.4). After 48 hours, samples were harvested.

Supernatant was aspirated, the culture was resuspended in remaining medium, 4 gl was added to 2 ml 0.01% glutaraldehyde, in PBS (pH 7.4). The erythrocytes were fixed at room temperature for 45 minutes and harvested by centrifugation at 2000 RPM for 15 minutes at 4 C. The supernatant was aspirated and parasites were stained in 2 ml propidium iodide (0.5 mg/ml) for at least 2 hours at 4 C. A fluorescence activated cell sorter (FACS) was used to quantitate fluorescent cells (twenty thousand cells per sample were counted). Experiments were performed in triplicate and on three separate occasions. The mean (SD) parasitemia for each sample was calculated and percent inhibition of invasion was calculated as follows: $f \text{ (SD. parasitemia for vehicle alone-SD parasitemia with sample)} * 100 / \text{SD parasitemia for vehicle alone}$. The FACS assay had previously been validated for accuracy by microscopy.

Protein binding and immunoblot analysis pl 15MSP-1 was incubated at 37 C for 30 minutes with 5 ul of human erythrocytes (30 days old, 5% hematocrit, washed 3 times with PBS (pH 7.4)) in a final volume of 30 u. l and layered on top of a 100 ul bed of silicon oil (=1.050, Aldrich #17, 563-3). Erythrocytes were pelleted at 10,000 x g for 10 minutes, leaving any soluble, unbound material in the aqueous phase. Both the aqueous phase and the silicon oil were removed and the pellet was washed 2 times with 1 ml of PBS (pH 7.4). The washed pellet was resuspended in 20 ul 1X SDS loading buffer, boiled and run on an 12% SD polyacrylamide gel. A Western blot analysis was performed as follows: Samples were transferred by electro-transfer onto ECL hybond nitrocellulose paper and blocked overnight, 5% milk in Western buffer (50 mM Tris (pH 7.4), 150 mM NaCl). The blot was incubated overnight with primary antibody in 5% milk, 0.2% Tween 20, in Western buffer, washed 3 times (Western buffer) and incubated with secondary antibody (donkey anti-rabbit Ig, horseradish peroxidase linked whole antibody, 1: 1,000, (Amersham)), in Western buffer, for 2 hours. After a final wash of 4 times (Western buffer), the blot was incubated with ECL western blotting detection reagents for 1 minute (Amersham) and developed on a Kodak X-OMAT AR scientific imaging film. For Fig. 1, panel B, an alkaline phosphatase labeled secondary antibody was used (same dilution as above) and after the final wash, 10 ml of a pre-stained substrate was added (Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega)) for 1-2 minutes and bands were visualized directly on the blot.

ELISA to measure human sera response to pl15MSP-1 A 96 well microtiter plate (IMMULON 2, Dynatech Laboratories) was coated with 100 u. l of pl15MSP-1 (15 ag/ml) in PBS (pH 7.4) and incubated overnight at room temperature. Wells were blocked with 200 p1 of 3% BSA in PBS (pH 7.4) for 5 hours at room temperature, washed 3 times with PBS (pH 7.4), incubated with 100 RI patient sera in varying concentrations for 3 hours at room temperature, washed 3 times with PBS (pH 7.4), incubated with 100 ul secondary antibody (sheep anti-human Ig, horseradish peroxidase linked whole antibody, 1: 2000 dilution in PBS (pH 7.4) (Amersham)) for 2 hours at room temperature, washed 4 times with PBS (pH 7.4), incubated with 100 ul 3, 3', 5,5'-tetramethyl benzidine (TMB) liquid substrate system (Sigma) for 30 minutes at room temperature, supplemented with 100 p1 0. 5M sulfuric acid and read on a 96 well plate reader at 450 nm.

Human serum samples Serum samples were taken from individuals native to Cameroon, Africa. All of the individuals are medical students ranging in age from late teens to late twenties and reside in Cameroon. Approximately 60-70% of the individuals are from the Bamileke tribe, in Western Cameroon. Patient history for individuals varied with regard to number of malaria infections and last occurrences of infection and disease symptoms. A vast majority of individuals failed to remember the last incidence of malaria and many take anti-malarial drugs upon symptoms of infection. Human serum used as controls was obtained from individuals never exposed to malaria. Patient sera kindly were provided by Dr. Armead Johnson, Director Immunogenetics, Department of Pediatric Clinical Services, Georgetown University.

Results Expression of pl SMSRI The pRSET over-expression construct containing the coding region for the 115 amino acid region of MSP-1 (1002-1115, West African Wellcome (Lagos) strain of *P. falciparum*) was sequenced and confirmed

as identical to previously published sequence data [Holder, et al., Nature 1985 ; 317: 270-3]. Over-expressed protein was purified to approximately 98% purity, as observed by SDS-PAGE (data not shown). Protein production gave final yields of approximately 1 mg of purified protein per 50 ml of induced bacterial culture. In attempts to remove the N-terminal histidine tag from pl 15MSP-1, the protein was treated with enterokinase (Biozyme), but nonspecific cleavage occurred. Therefore, the pl 15MSP-1 protein used throughout this study contains the histidine tag and intervening vector sequences from the over-expression construct, resulting in a protein with a theoretical molecular mass of 18,475 Daltons.

SDS-PAGE analysis of pl 15MSP-1 resulted in an apparent molecular mass of approximately 20 kDa, slightly more than the theoretical value (data not shown). This slight difference may be due to anomalous migration during SDS-PAGE. Confirmation of the origin of pl 15MSP-1 was further ascertained by performing a Western blot using polyclonal antibodies raised to a 65 kDa C-terminal region of MSP-1 from work previously performed in our laboratory [Yang, et al., Vaccine 1997; 15: 1303-13]. Polyclonal antibodies to this region, which fully contains pl 15MSP-1, identified a single protein of approximately 20 kDa (data not shown). These data indicate that the over-expressed and purified protein is pl 15MSP-1.

To confirm protein identity further, partial N-terminal sequencing of pl 15MSP-1 was performed. Due to unsuccessful enterokinase cleavage, partial sequencing resulted in identification of the start codon and intervening vector sequences of the construct (data not shown). However, these data do confirm that pl 15MSP-1 is an over-expressed protein, generated by the expression vector, and not a non-specific, bacterial protein of similar size.

Verification of pII5MSP-1 antibodies Polyclonal antibodies to pl 15MSP-1 were produced and purified on a protein A-agarose column to a final concentration of 3.1 mg/ml (dialyzed in PBS (pH 7.4)) with a final titer between 1: 80,000 and 1: 100,000 (data not shown). The high titer observed in the rabbit serum illustrates the high antigenic properties of this region of MSP-1. Western blots were performed using purified pII5MSP-1 to successfully confirm the specificity of these antibodies. In addition, pl 15MSP-1 antibodies were used to perform Western blots against the previously described 65 kDa C-terminal region of MSP-1 and full length MSP-1 isolated from late trophozoite/early schizont stage infected erythrocytes (Fig. 1). Panel A shows the recognition of full length, 195 kDa MSP-1 (lanes 2-4) of increasing concentration (1,5 and 10 µg of lysed infected erythrocytes (5% hematocrit)) by pl 15MSP-1 antibodies. A control sample of uninfected erythrocytes is shown in lane 1. Panel B shows the recognition of the 65 kDa 3' terminus of MSP-1 by pl 15MSP-1 antibodies. Positive identification of the 65 kDa and 195 kDa full length MSP-1 confirm the identity and specificity of pl 15MSP-1 antibodies.

Inhibition of parasite invasion by pII5MSP-1 antibodies, in vitro In vitro invasion assays were performed to determine the ability of pl 15MSP-1 antibodies to inhibit parasite invasion (Fig. 2). Invasion assays test the ability of an antibody or compound to inhibit parasite re-invasion. Parasitic invasion is allowed to proceed under in vitro conditions in the presence and absence of antibody. As erythrocytes burst and parasites re-invade fresh erythrocytes, parasite re-invasion will be diminished, if the antibody in the sample exerts an inhibitory effect. Results from experimental samples can be compared to controls: pre-immune rabbit serum and vehicle alone, i. e. PBS (pH 7.4) to determine the magnitude of inhibition of invasion. Experiments were performed in triplicate and repeated at least three times.

When compared to values of pre-immune rabbit sera samples, the data show that parasite invasion is drastically inhibited in the presence of pl 15MSP-1 antibodies.

Furthermore, a dose dependent response is seen, ending in a maximum effective concentration. Positive controls were samples of heparin (0.2 mg/ml), a highly poly-anionic compound [Clark and Davidson, Glycoconj J. 1997; 12: 473-9] and EGTA (5 mM), both of which have been shown to completely inhibit parasite invasion, in vitro [McCallum-Deighton and Holder, Mol Biochem Parasitol. 1992; 59: 1-14]. These two positive controls resulted in 90-95% inhibition. Levels of 100% are difficult to achieve due to background fluorescence intrinsic to the system and of erythrocytes that have not yet burst and contain fluorescent schizonts. These data illustrate that p 115MSP-1 antibodies are highly successful in abrogating parasite invasion and suggest that this 115 amino acid region of MSP-1 is significant during the parasitic recognition/invasion process.

Inhibition of parasite invasion by purified pII5MSP-1 protein, in vitro During the in vitro invasion assay, antibodies to pl 15MSP-1 bind to their specific domain on the parasitic cell surface-a domain within the full length MSP-1. It is unknown whether antibodies recognize the pl 15MSP-1 epitopes immediately after erythrocytic bursting, or at some point during the invasion process; however, an effective inhibition of invasion is seen. To further investigate the molecular interactions

between parasite and erythrocytes, we performed the corollary experiment to the invasion assay using pl 15MSP-1 antibodies. This experiment tested whether or not competition by pl 15MSP-1 for a putative erythrocyte cell surface receptor could prevent invasion. In vitro invasion assays were performed using varying concentrations of purified pl 15MSP-1 (Fig. 3).

Results show a dose dependent inhibition of invasion by pl 15MSP-1, with significant levels of inhibition achieved at higher protein concentrations. Experiments were performed in triplicate and repeated at least three times; variation within each experiment and between experiments was minimal. A variety of control experiments was performed to eliminate certain properties of pl 15MSP-1 as the cause of abrogation of parasite invasion. Invasion assays were performed with vast molar excesses of the following proteins: 1) histidine tagged dnaA protein, generated in the same over-expression system as pl 15MSP-1, 2) equine myoglobin, a protein of similar size to pl 15MSP-1 and 3) cytochrome C, a very basic protein and similar in charge to pl 15MSP-1. All of these control experiments resulted in baseline levels of invasion inhibition (data not shown), indicating that results with purified pl 15MSP-1 can be attributed to the amino acid sequence of the protein and not to its histidine tag, size or charge.

In previous experiments, antibodies to pl 15MSP-1 recognized corresponding epitopes within the p38 fragment of MSP-1 and prevented parasite invasion. In contrast, this experiment shows that pl 15MSP-1 can prevent parasite invasion by competing with merozoites for a specific erythrocytic cell surface receptor. These data confirm and further substantiate the functional importance of this region of MSP-1 in the parasitic recognition/invasion process. pl 15MSP-1 binding to human erythrocytes, *in vitro*. In order to investigate the potential molecular interactions between pl 15MSP-1 and erythrocytes suggested in the previous experiments, we performed a series of *in vitro* binding assays. The theory behind these binding experiments is that after pl 15MSP-1 is allowed to incubate with erythrocytes, centrifugation through a bed of silicon oil sediments the erythrocytes and any bound material (all unbound material remains in the soluble phase above the silicon oil). A final wash is performed and specifically bound protein and samples are analyzed by SDS-PAGE. Western analysis is performed using pl 15MSP-1 antibodies.

This experimental design ensures that bound protein is separated from unbound protein and that contamination from soluble, unbound protein does not occur. Results of binding reactions between erythrocytes and pl 15MSP-1 are shown in a Western blot using pl 15MSP-1 specific antibodies (Fig. 4).

The results indicate that p115MSP-1 binds to human erythrocytes (lane 3).

Erythrocytes that underwent the same experimental procedure as the sample show no band at 20 kDa (lane 2). Purified pl 15MSP-1 is shown in lane 1 and confirms that the bound protein in lane 1 is indeed purified pl 15MSP-1. These results indicate that pl 15MSP-1 binds to human erythrocytes with a high degree of specificity. p115MSP-1 binding to EnA (-) erythrocytes, *in vitro*. It has been hypothesized that the erythrocyte cell surface glycoprotein glycophorin A plays an important role in parasite recognition/invasion process [Miller, et al., J Exp Med 1977; 146: 277-81 ; Pasvol, et al., Nature 1982 ; 297: 64-6; and Perkins M., J Cell Biol 1981; 90: 563-7]. To test whether or not p115MSP-1 binds to glycophorin A, we obtained erythrocytes genetically devoid of this cell surface protein. EnA (-) erythrocytes are identical to normal, human erythrocytes except for the lack of glycophorin A. This phenotype occurs with a very low frequency [Holder, et al., Parasite Immunol. 1988; 10: 607-17]. Individuals lacking glycophorin A are much less susceptible to parasitic invasion and invasion is decreased by approximately 52-92% of control levels [Perkins, *supra*]. It is important to note that even in the absence of glycophorin A, the malaria parasite is still capable of invasion, suggesting the participation of other as yet unidentified erythrocyte cell surface components.

EnA (-) erythrocytes were obtained from the New York Blood Center, where they had been previously tested to confirm the absence of cell surface glycophorin A.

Binding assays using pl 15MSP-1 and EnA (-) erythrocytes (Fig. 5) were performed.

Results indicate that pl 15MSP-1 is capable of binding to EnA (-) erythrocytes (lane 3) when compared to control samples of EnA (-) erythrocytes alone (lane 2). Purified pl 15MSP-1 is shown in lane 1 and migrates at the same position as bound material. These results suggest a possible additional (i. e. non-glycophorin dependent) protein-protein interaction between MSP-1 and erythrocytes.

The autoradiograms for the Western blot results shown in Figures 4 and 5 were scanned and analyzed by densitometry to determine the extent of binding of pI 15MSP-1 to erythrocytes versus that of EnA (-) erythrocytes. The numerical values obtained for the control lane of pI 15MSP-1 alone were out of the linear range of detectability; therefore, percent of binding was not calculated. However, absolute values obtained for the binding of pI 15MSP-1 to normal erythrocytes and EnA (-) erythrocytes were very similar, suggesting the presence of a common receptor on the two cell types.
 <P> Recognition of pI 15MSP-1 by human sera from individuals in malaria-endemic regions. Individuals who live in malaria-endemic areas develop clinical immunity to malaria over time and it has been suggested that antibodies play a role in protection against disease [Yang, et al., Vaccine 1997; 15: 1303-13]. To address whether or not antibodies to pI 15MSP-1 are part of the human immune response, we conducted experiments testing the ability of sera from individuals living in a malaria-endemic area to recognize pI 15MSP-1. ELISA studies were performed on 61 serum samples and a scatter graph of the absorbance values was plotted (Fig. 6).

When compared to human serum from uninfected individuals, the data show a varying degree of positive responses to pI 15MSP-1, with 62% considered as a positive recognition (cut off value of 2.5X control value). These data indicate that individuals who have been infected with malaria are able to produce antibodies to pI 15MSP-1, *in vivo*. The sera we tested were incomplete with regard to patient histories, with the majority of individuals not remembering their last infection of malaria (implying that life-time infection rate among this sample pool may have been low). Studies are underway utilizing sera from children, older adults and individuals with high titer to MSP-1. To corroborate the ELISA results, we performed native dot blot experiments (data not shown) on six selected samples (both positive and negative); these sets of experiments confirmed results seen by ELISA.

The goal of the analysis of human serum reactivity to pI 15MSP-1 was to see whether or not antibodies to this region are made *in vivo*. When coupled with previous data from this study, these data not only suggest that an immune response to pI 15MSP-1 occurs in humans, but also implicate a potential role for pI 15MSP-1 antibodies in the clearance of the malaria pathogen and/or prevention of disease.

4. Discussion It had been previously shown that a 115 amino acid region of MSP-1 of *P. falciparum*, pI 15MSP-1, may be involved in parasite recognition/invasion of human erythrocytes. Our objective in this study was to further investigate this region, identifying its potential role in invasion and whether or not it could function as an antigen and elicit the production of functional, inhibitory antibodies.

The binding studies performed in these examples clearly illustrate that this region of the p38 domain of MSP-1 can bind specifically to human erythrocytes. This binding interaction also appears to be sialic acid (N-acetylneurameric acid) independent, based on a positive binding results seen with neuraminidase treated erythrocytes and pI 15MSP-1 (data not shown). The ability of pI 15MSP-1 to bind to glycophorin A negative erythrocytes suggests a novel receptor on the erythrocyte cell surface. A binding interaction, whether initial attraction between parasite and erythrocyte, close range stabilization or a signal initiation event, is a significant step for effective parasite invasion. The specificity of this binding interaction can be addressed in several additional ways. The fact that antibodies to pI 15MSP-1, and pI 15MSP-1 itself, inhibit parasite invasion illustrates the functional specificity of pI 15MSP-1 and its role in the parasitic recognition/invasion process.

Furthermore, binding experiments were performed in the presence of vast excesses of equine myoglobin and whole cell extract from BSC-1 cells (to introduce an excess of proteins of varying molecular mass); in all of the experiments, pI 15MSP-1 binding was not abrogated by the presence of these compounds (data not shown).

A major focus of research on parasite binding has been on the adherence of erythrocytes to the endothelial lining of blood vessels; however, binding of merozoites to erythrocytes is a crucial event for parasite survival. In this study, the exact components involved in the interaction described still need to be further elucidated, specifically in terms of the erythrocytic receptor. However, in terms of pI 115MSP-1, when results from the binding assays are correlated with results from the invasion assays, an argument can be made for the necessity of pI 115MSP-1 for optimal invasion.

Analysis of the primary amino acid sequence of pI 15MSP-1 reveals several interesting features of this region. Not only is the sequence very basic in nature, with an overall +7 positive charge and a theoretical pI of 9.44, but 43% of the amino acids can be considered highly hydrophilic [Biochemistry, 2nd ed.. New Jersey: Neil Patterson Publishers, Prentice Hall 1994]. When considered in context of the intact p38 fragment, pI 15MSP-1 is by far the most basic region. The theoretical

pI of p38 as a whole is 5.63 and the theoretical pI of p38 without the p 115MSP-1 sequence is 4.83. These facts may suggest that the vast majority of the 115 amino acids of p38 are found on the exterior of p38, while the remaining 5'and 3'hydrophobic flanking sequences comprise the interior of the properly folded p38 fragment.

Preliminary data indicate that sera of individuals from malaria-endemic regions are unable to recognize denatured pI 15MSP-1 by SDS-PAGE (data not shown). When taken into consideration with the ELISA and dot blot data presented in this study, it appears that a native, conformational structure of pI 15MSP-1 is needed for proper antibody recognition.

Therefore, binding between pI 15MSP-1 and erythrocytes is most likely dependent on proper pI 15MSP-1 conformation (secondary and tertiary structure) and a further delineation of the exact binding sequence may not provide much more information. In fact, a study by Patarroyo, et al. focused on binding interactions between erythrocytes and a set of 78 synthetically engineered peptides spanning MSP-1 [Urquiza, et al., Parasite Immunology.

1996; 18 : 515-26]. Results indicated several regions of binding that modestly inhibited invasion (< 60%), *in vitro*. It is important to note that in this study no peptide within the pI 15MSP-1 domain was identified as significant for binding and thus, antibodies were never generated for testing in invasion assays. Peptides investigated in that study, however, were only 20 amino acids long and this length is most likely insufficient to create the secondary, and possibly tertiary, structure necessary for binding to erythrocytes. Our study evidences that pI 15MSP-1 antibodies are more effective at inhibiting parasite invasion than polypeptides of 20 amino acids, and suggests that pI 15MSP-1 is of large enough size to create structural domains similar to those found in full length MSP-1 and responsible for erythrocytic binding.

It can be envisioned that as merozoites are released from a bursting erythrocyte, there is an initial long range interaction with erythrocytes. This results in a binding reaction and eventual parasite reorientation and invasion. Since electrostatic interactions can act over relatively long distances, the extremely basic region of p 11 SMSP-1 may be involved in a charge mediated, long distance binding interaction with receptors on the erythrocyte cell surface. It can be hypothesized that once a long distance interaction has occurred, other parasite proteins, such as EBA175 (Fig. 6), which has been shown to bind to glycophorin A, or other MSP-1 processed polypeptides, stabilize the parasite/erythrocyte bond and allow for proper invasion.

A major hurdle in development of a malaria vaccine has been the identification of regions that are both highly antigenic and functionally relevant. Indeed studies have shown that antibodies produced *in vivo* against non-critical regions of parasite cell surface proteins can block effective antibodies from binding [Patino, et al; J Exp Med 1997; 186 : 1689-99].

The ability of pI 15MSP-1 antibodies to prevent parasite invasion at the high levels seen in this study suggests the importance of this region during invasion. Levels of inhibition approaching 90% are similar to positive control values (EGTA and heparin). Microscopy of samples post-invasion reveals a small percentage of late stage schizonts which results in nonspecific, aberrant fluorescence and lowers the threshold of maximal inhibition, for both samples and positive controls. In pI 15MSP-1 antibody samples, as well as controls, no ring stage parasites could be seen by microscopy, indicating effective inhibition of invading merozoites. This suggests that at higher concentrations pI 15MSP-1 antibodies are close to 100% effective in inhibiting parasite invasion.

The fact that pI 15MSP-1 itself inhibits invasion further illustrates the importance of a pI 15MSP-1/erythrocyte interaction for efficient invasion. The inhibitory effects of the protein itself approach 70% in magnitude, a highly significant level. This abrogation of invasion suggests that p 115MSP-1 is competing with MSP-1 on invading merozoites for a specific erythrocyte receptor. This erythrocyte cell surface protein still needs to be identified, but several candidates exist, including: bands 3 and 7, PAS-1,2,3 or 4. *In vitro* invasion assays attempt to simulate parasitic invasion, however many caveats exist. The static nature of the system creates a far more favorable environment for invasion than the fast moving, multi-component environment of the blood stream. Therefore, a long distance interaction between merozoites and erythrocytes may not be as necessary in an *in vitro* situation as it is *in vivo*. The ability of both pI 15MSP-1 and its respective antibodies to inhibit invasion provides strong evidence for the functional importance of this region during parasite invasion.

The high antigenicity of pI 15MSP-1 (titer of between 1: 80,000 and 1: 100,000) further substantiates this region as a vaccine component. Polyclonal antibodies initially raised to this region resulted in inhibition levels of only 55% and 33% at

antibody concentrations of 200 ug and 100 u. g, respectively (unpublished data). However, the antibody titer of p 115MSP-1 antibodies in those experiments was only between 1: 10,000 and 1: 15,000, a 6-8 fold lower titer than antibodies used in the experiments presented here. The correlation between higher titer of antibody and greater inhibition illustrates the specificity of p 115MSP-1 antibodies against invading merozoites.

Malaria has an annual death rate of 1-2%, with the majority of infections cleared by a natural host immune response. A variety of immune components have been suggested to play roles in effective clearance of malaria, including both B-cell and T-cell activating epitopes [Riley, et al., Parasitology Today 1991; 7: 5-10]. Functional immunity occurs in a large number of individuals living in endemic areas and suggests that although infection can be established, disease symptoms may be abrogated by better clearance of merozoites after erythrocytic bursting [Doolan and Hoffinan, Parasitology Today 1997; 13: 171-8; and Berman and Stelcetee, Public health and preventive medicine 1992: 240-52]. Identification of antigenic regions responsible for prevention of re-invasion and/or clearance of uninvaded merozoites is important for development of a multi-subunit vaccine.

An antigenic domain may be extremely effective in vitro, but if antibodies to this region are not produced in infected individuals, chances of this region playing an important role in clearance and/or re-invasion of merozoites is unlikely. For this reason, the recognition of p 115MSP-1 by sera from individuals living in malaria-endemic regions is a crucial and unexpected observation that ties together the data in this study. The fact that this fragment is recognized by 62% of the sera tested indicates that this region should be immunogenic, *in vivo*. (Note that the percentage of sera that positively identify pi 15MSP-1 would be higher if the cut-off value was lowered to an acceptable 2 times control.) Antibodies to MSP-1 have been identified in greater than 74% of the sera of individuals between ages 15-29 living in malaria-endemic regions. The fact that the individuals tested in this study had spent much time in urban settings and had taken anti-malarial drugs when taken ill may provide explanation for the lower value of positive responses.



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(WO/2001/085927) MALARIA VACCINE AND METHODS THEREOF BASED UPON NOVEL ANTIGENIC DOMAIN OF *PLASMODIUM FALCIPARUM*

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Claims 1. A vaccine comprising an expression vector comprising a nucleotide sequence which encodes for an immunogenic p1 15MSP-1 peptide or immunogenic fragment thereof in vaccine dosage form in combination with a pharmaceutically acceptable excipient, carrier or additive.

2. The vaccine according to claim 1 wherein said nucleotide sequence also encodes for a mammalian signal sequence or mammalian anchor sequence.

3. The vaccine according to claim 2 wherein said signal sequence is a human signal sequence.

4. The vaccine according to claim 2 wherein said anchor sequence is a human anchor sequence.

5. The vaccine according to claim 2 wherein said signal sequence is a human signal sequence and said anchor sequence is a human anchor sequence.

6. The vaccine according to claim 1 which also encodes for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

7. The vaccine according to claim 2 which also encodes for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

8. The vaccine according to claim 5 which also encodes for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

9. The vaccine according to claim 1 wherein said expression vector is obtained from a Deficient Venezuela Encephalitis Equine virus.

10. A method of vaccinating a patient against malaria comprising administering an effective amount of a recombinant viral vector capable of expressing P 11 SMSP-1 peptide after administration of said vaccine to a patient.

. The method according to claim 10 wherein said vector is a DVEE viral vector.

12. The method according to claim 10 wherein said nucleotide sequence also encodes for a mammalian signal sequence or mammalian anchor sequence.

13. The method according to claim 12 wherein said signal sequence is a human signal sequence.

14. The method according to claim 12 wherein said anchor sequence is a human anchor sequence.

15. The method according to claim 12 wherein said signal sequence is a human signal sequence and said anchor

sequence is a human anchor sequence.

16. The method according to claim 10 wherein said vector also encodes for an immunogenic peptide selected from the group consisting of PI 9, rap-1, resa, ebp- 175, gbp-130, csp 1, csp 2 and pid25.

17. The method according to claim 11 wherein said vector also encodes for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp- 175, gbp-130, csp 1, csp 2 and pfd25.

18. The vaccine according to claim 5 which also encodes for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

19. A chimeric peptide sequence comprising : a) an immunogenic P115MSP-1 peptide and at least one additional peptide selected from the group consisting of an anchor sequence peptide, a signal sequence peptide, a second immunogenic peptide or mixtures thereof.

20. The peptide according to claim 19 wherein said signal sequence is a human signal sequence.

21. The peptide according to claim 19 wherein said anchor sequence is a human anchor sequence.

22. The peptide according to claim 19 wherein said signal sequence is a human signal sequence and said anchor sequence is a human anchor sequence.

23. The peptide according to claim 19 wherein said second immunogenic peptide is selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

24. A method of vaccinating a patient comprising administering to said patient an effective amount of a chimeric peptide according to claim 19 to said patient.

25. A method of vaccinating a patient comprising administering to said patient a DNA sequence which encodes for P115MSP-1 peptide.

26. The method according to claim 25 wherein said DNA sequence further encodes for a second immunogenic protein selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2, pfd25 peptide and mixtures thereof.

27. The method according to claim 25 or 26 wherein said DNA sequence further encodes for a signal protein, an anchor protein or a signal protein and an anchor protein.

28. An expression vector comprising a nucleotide sequence which encodes for an immunogenic P115MSP-1 peptide and a mammalian signal sequence or a mammalian anchor sequence.

29. The vector according to claim 26 wherein said vector is a DVEE viral vector.

30. The vector according to claim 26 wherein said signal sequence is a human signal sequence.

31. The vector according to claim 26 wherein said anchor sequence is a human anchor sequence.

32. The vector according to claim 26 wherein said signal sequence is a human signal sequence and said anchor sequence is a human anchor sequence.

33. The vector according to claim 26 further encoding for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

34. The vector according to claim 27 further encoding for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

35. The vector according to claim 28 further encoding for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

36. A method of vaccinating a patient against malaria comprising administering to said patient in need thereof a vaccine comprising an effective amount of pl 15MSP-1 peptide in vaccine dosage form in a pharmaceutically acceptable carrier, excipient or additive.

37. The method according to claim 34 wherein said vaccine further comprises an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp- 175, gbp-130, csp 1, csp 2 and pfd25.

38. A method of vaccinating a patient from the risk of infection by *P. falciparum* said method comprising administering to said patient an effective amount of a chimeric protein comprising pl 15MSP-1 peptide in combination with at least one targeting peptide.

39. The method according to claim 38 wherein said chimeric protein comprises at least one additional immunogenic peptide selected from the group consisting of PI 9, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

40. A method of vaccinating a patient against infection by *P. falciparum* said method comprising administering to said patient an effective amount of a DNA sequence which encodes for a chimeric protein comprising pl 15MSP-1 peptide in combination with at least one targeting peptide.

41. The method according to claim 40 wherein said chimeric protein further comprises at least one additional immunogenic peptide selected from the group consisting of PI 9, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

AMENDED CLAIMS [received by the International Bureau on 15 October 2001 (15.10.01); original claim 36 amended; new claim 42 added : remaining claims unchanged (2 pages)] for a second immunogenic protein selected from the group consisting of PI 19, rap-1, esau ebb gbp-130, csp 1, csp 2, pfd25 peptide and mixtures thereof.

27. The method according to claim 25 or 26 wherein said DNA sequence further encodes for a signal protein, an anchor protein or a signal protein and an anchor protein.

28. An expression vector comprising a nucleotide sequence which encodes for an immunogenic P115MSP-1 peptide and a mammalian signal sequence or a mammalian anchor sequence 29. The vector according to claim 26 wherein said vector is a DVEE viral vector.

3c. The vector according to claim 26 wherein said signal sequence is a human signal sequence.

31. The vector according to claim 26 wherein said anchor sequence is a human anchor sequence.

32. The vector according to claim 26 wherein said signal sequence is a human signal sequence and said anchor sequence is a human anchor sequence.

33. The vector according to claim 26 further encoding for an immunogenic peptide selected from the group consisting of ? 19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

34. The vector according to claim 27 further encoding for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

35. The vector according to claim 28 further encoding for an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

36. A method of vaccinating a patient against malaria comprising administering to said patient in need thereof a vaccine comprising an effective amount of p115MSP-1 peptide or an immunogenic fragment thereof in vaccine dosage form in a pharmaceutically acceptable carrier, excipient or additive, optionally in the presence of an adjuvant.

37. Hrhe meCod according to claim 34 wherein said vaccine further comprises an immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, bp-130, csp 1, csp 2 and pfd25.

38. A method of vaccinating a patient from the risk of infection by *P. falciparum* said method comprising administering to said patient an effective amount of a chimeric protein comprising p115MSP-1 peptide in combination with at least one targeting peptide.

39. The method according to claim 38 wherein said chimeric protein comprises at least one additional immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

40. A method of vaccinating a patient against infection by *P. falciparum* said method comprising administering to said patient an effective amount of a DNA sequence which encodes for a chimeric protein comprising p115MSP-1 peptide in combination with at least one targeting peptide.

41. The method according to claim 40 wherein said chimeric protein further comprises at least one additional immunogenic peptide selected from the group consisting of P19, rap-1, resa, ebp-175, gbp-130, csp 1, csp 2 and pfd25.

42. p115MSP-1 peptide or an immunogenic fragment thereof.